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TECHNICAL MANUSCRIPT 264

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IMMUNOCHEMICAL AND BIOLOGICAL ACTIVITY  
OF STAPHYLOCOCCAL ENTEROTOXIN

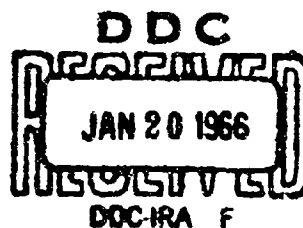
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BIOLOGICAL LABORATORIES  
FORT DETRICK

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U.S. ARMY BIOLOGICAL LABORATORIES  
Fort Detrick, Frederick, Maryland

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EFFECT OF FORMALIN ON THE IMMUNOCHEMICAL AND BIOLOGICAL ACTIVITY  
OF STAPHYLOCOCCAL ENTEROTOXIN

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

# ABSTRACT

Purified staphylococcal enterotoxin B was treated with formalin at 37 C and pH 7.3. The effect of formalin concentration and duration of exposure was studied by immunochemical and biological means. By 8 hours, toxin treated with 0.15 or 0.30% formalin (v/v) lost some of its ability to precipitate antibody. At 48 hours the toxoid behaved as a cross-reacting antigen in that an antibody excess occurred throughout the range of the quantitative precipitin titrations and the zone of antibody excess was followed by a zone in which both antigen and antibody were in excess. In the Ouchterlony double gel diffusion test after 48 hours, toxoid gave a reaction of partial identity when compared with toxin. When tested by immunoelectrophoresis at pH 6.0, 0.1 M  $\text{PO}_4$  buffer, the formalin-treated toxin migrated further toward the cathode than did the toxin.

Toxin treated for about 48 hours with 0.1 to 0.3% formalin maintained its emetic activity unimpaired when injected into monkeys intravenously; however, the amount required to cause death increased more than thirty-fold from 25 to more than 750  $\mu\text{g}$  per kg body weight. Emetic activity decreased after 48 hours' treatment and appeared to be completely lost after 9 days. Toxoid also lost its pyrogenic activity for rabbits.

EFFECT OF FORMALIN ON THE IMMUNOCHEMICAL AND BIOLOGICAL ACTIVITY  
OF PURIFIED STAPHYLOCOCCAL ENTEROTOXIN

Formalin has long been used in immunology to inactivate toxins. Most of the data that are available indicate little or no change in the antigenic properties following treatment with this reagent. This study was performed in an attempt to correlate changes in biological and immunochemical activity of purified staphylococcal enterotoxin B.

Formaldehyde (HCHO) reacts primarily with the imino and amino groups of proteins and amino acids forming methylol amine,  $\text{NH}\cdot\text{CH}_2\text{OH}$ . The aldehyde will also react with other side chains (depending upon conditions of the reaction) such as amide, guanidyl, indol, and  $\text{CH}_2$ . Methylol amine will form cross-linkages with other  $\text{NH}_2$  groups and other side chains. Toxoid formation does not parallel reaction between  $\text{NH}_2$  groups and HCHO as followed by Sorenson's formal titrations.

Reaction with diphtheria and other toxins has been shown to occur without affecting the ability of the toxin to flocculate with antibody or without an effect on the antigenic properties.<sup>1,2</sup> Ricin, however, resists complete detoxification with HCHO. Fisher<sup>3</sup> reported that HCHO reduced the immunizing activity of hemolysins and neutralizing toxins in crude staphylococcal culture filtrates.

Staphylococcal enterotoxin B (1 mg/ml) prepared according to the procedure of Schantz et al.<sup>4</sup> and free of hemolysins, apyrase, and other metabolic products, was exposed to various concentrations of formalin for varying lengths of time at 37 C and pH 7.3. Subsequent to exposure to formalin, excess reagent was removed either by extensive dialysis against large volumes of 0.02 M phosphate-buffered saline, pH 7.3, or by reacting the excess formalin with  $\text{NaHSO}_3$ . It was determined previously that this compound had no effect on the activity of the toxin.

The changes in the immunochemical activity were measured by (i) the quantitative precipitin test of Heidelberger and Kendall,<sup>5</sup> (ii) single gel diffusion in tubes, (iii) double gel diffusion in plates, and (iv) immunoelectrophoresis. The antiserum used in all of these tests was a pool of sera from rabbits intensively immunized with the purified toxin.

The quantitative precipitin test of Heidelberger and Kendall was performed by incubating the toxin-antitoxin mixtures at 37 C for 4 hours followed by 4 C for 2 to 4 days. The precipitates were washed, dissolved in 0.25 M acetic acid, and the protein nitrogen was determined spectrophotometrically at 277 mμ. Immunoelectrophoresis was performed with LKB\* apparatus using 0.1 M phosphate buffer, pH 6.0, and 50 milliamperes.

\* LKB Instruments, Inc., Rockville, Maryland.

Biological activity was determined by observing emesis and/or death in Macaca mulatta monkeys following intravenous injection. The monkeys weighed from 2.0 to 3.5 kg with the majority of them weighing 2.5 to 3.0 kg. The animals were observed 5 hours for emesis and held for 10 days to determine the lethal effect of high doses of toxin.

Figure 1 shows the effect of concentration of HCHO varying from 0.6 through 1.4% (v/v) on the precipitin reaction after exposure of the toxin for 6 days at 37 C. With increasing concentrations of formalin the toxin became less capable of precipitating the antitoxin completely. The equivalence zone for the untreated toxin was between 6.0 and 11.0  $\mu$ g toxin N; that for the toxoid was greater than 24.1  $\mu$ g toxin N in all cases. The graph also contains data that give evidence of partial destruction of the activity of the antibody by free formalin when toxin treated with 0.6% HCHO was tested before and after dialysis. Others<sup>6,7</sup> have shown that formalin-treated antibody can combine with antigen but loses its ability to precipitate. In the agar diffusion tests, there was little or no change in the rate of diffusion of the toxoids prepared with the different amounts of HCHO when compared with untreated toxin. The precipitate bands, however, were lighter in density and more difficult to measure. In the Ouchterlony plate a "reaction of partial identity" was observed between toxin and toxoid. The toxoids produced either (i) double bands that both fused partially with the adjacent single band produced with toxin, or (ii) in some cases, (Figure 2) a single diffuse band that partially fused with the toxin band. The toxoids prepared with 0.6 to 1.4% HCHO after 6 days were no longer capable of inducing emesis when 10  $\mu$ g/kg were injected into monkeys, whereas the ED<sub>50</sub> of the control toxin was from 0.1 to 0.3  $\mu$ g/kg.

The effect of 0.15% and 0.30% HCHO on enterotoxin over a time period ranging from 0 hour through 32 weeks was also investigated. With both concentrations of the aldehyde a loss of precipitating activity was observed within 8 hours. This loss of activity increased with time (Figure 3) but there was still some activity remaining after 32 weeks. Changes in the pattern of the precipitate band in the gel diffusion plate (as described above) appeared at 48 hr.

The "reaction of partial identity" on the agar diffusion plates, the disappearance of an equivalence zone in the precipitin test, and the appearance of a zone in which both antigen and antibody were present in excess indicated that HCHO had altered the protein so that it behaved either as a cross-reacting antigen or a mixture of antigens.

Within 3 to 5 days of incubation at 37 C with 0.3% formalin the toxin lost its pyrogenic activity for rabbits. Whereas as little as 25  $\mu$ g of untreated toxin induced an increase in fever of 2 to 4 F, 1.9 mg of the toxoid caused no hyperthermia.

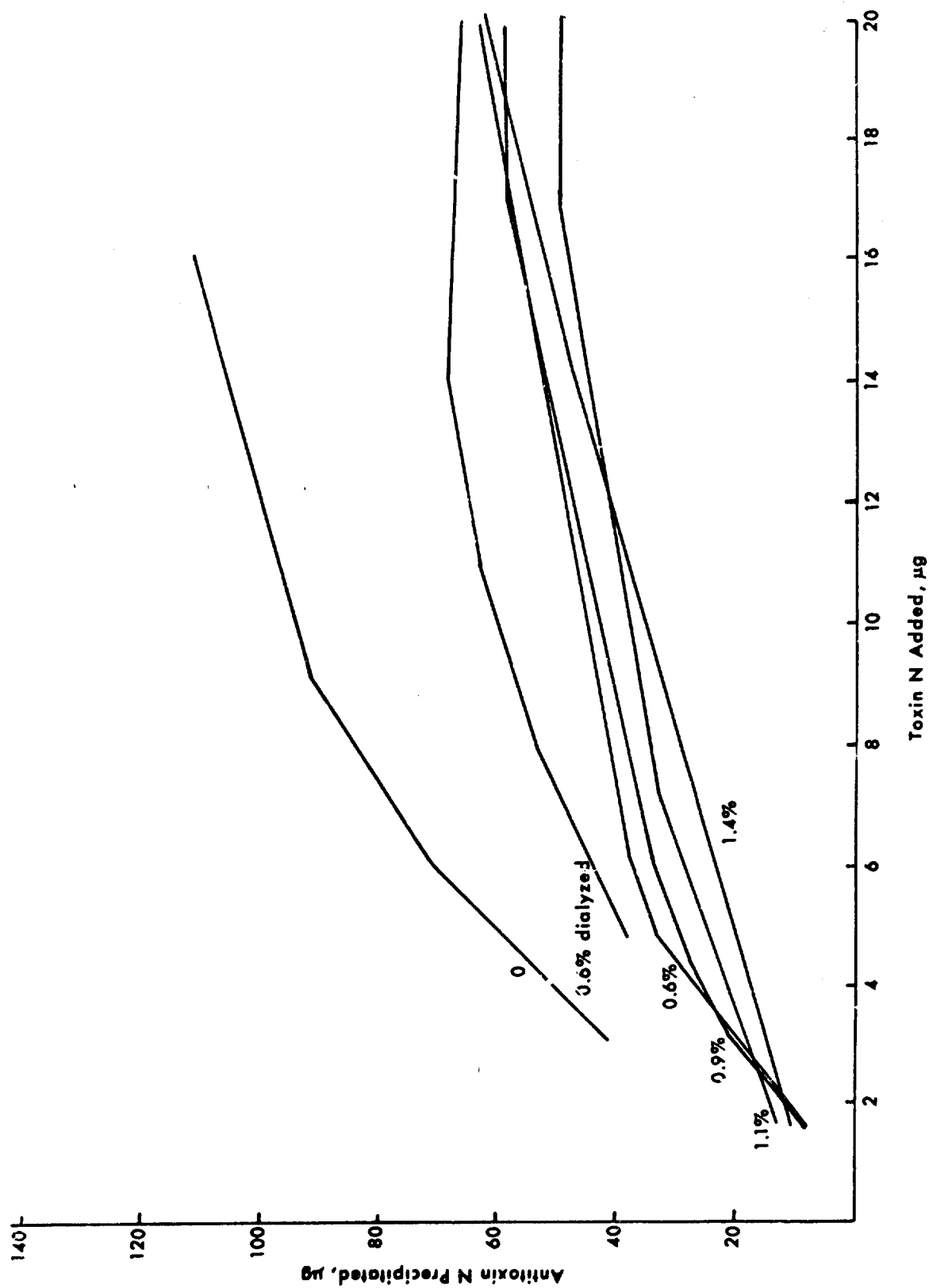


Figure 1. The Effect of Formalin on the Immunochemical Activity of Enterotoxin B; Precipitation Curves of Antitoxin b and Toxin Treated with Various Concentrations of Formalin.

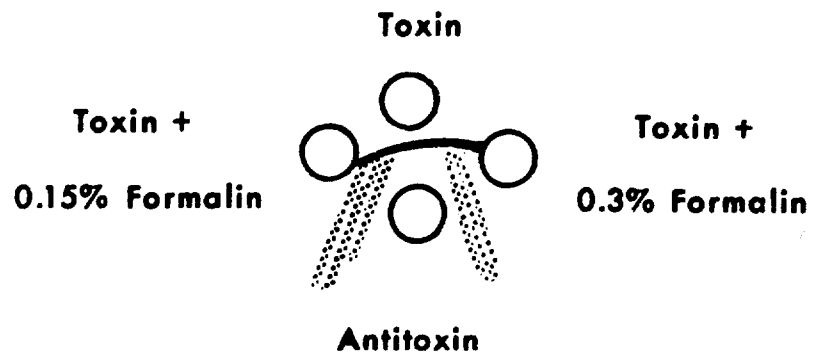


Figure 2. Comparison of the Reaction of Enterotoxin B and Enterotoxin B with Antitoxin.

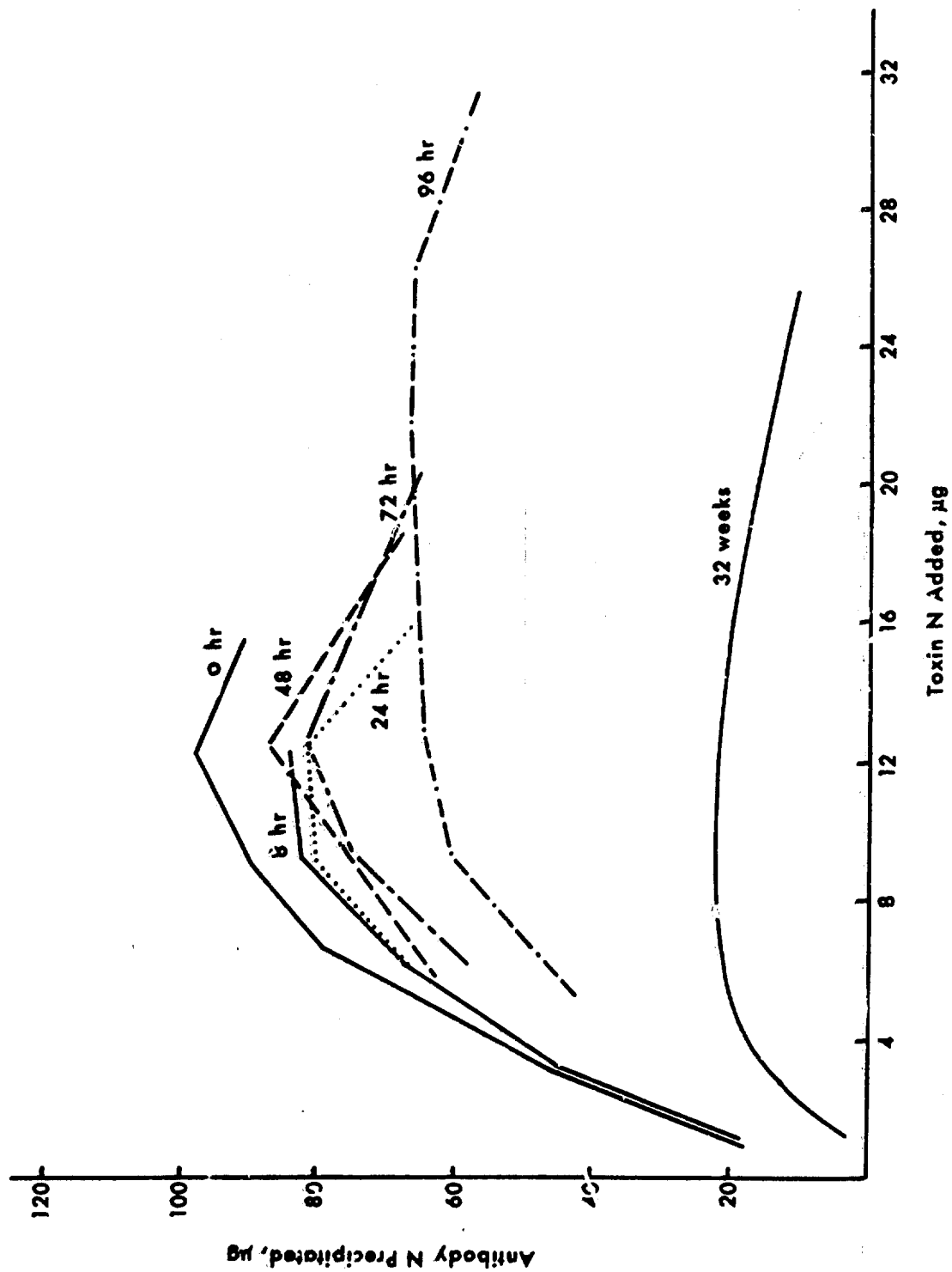


Figure 3. Effect of 0.15% HCHO on Immunochemical Activity of Enterotoxin B.

When antitoxin and antitoxoid were compared in the precipitin test using the homologous or heterologous systems, very little difference was noted. Toxin precipitated slightly more antibody nitrogen from antitoxin than did toxoid and, conversely, toxoid precipitated slightly more anti-toxoid antibody than did toxin; on the Ouchterlony plate no evidence of cross reactions was observed.

When studied by immunoelectrophoresis in pH 6.0, 0.1 M buffer the toxoid moved further toward the cathode than did the untreated toxin. The precipitate band no longer formed a well-defined arc but was flat and extended.

When injected into the femoral vein of Macaca mulatta monkeys, the toxin treated with 0.10 to 0.3% formalin for 8 to 24 hours showed no loss in its ability to induce emesis. There was, however, an increase in the LD<sub>50</sub> value from 24 µg per kg body weight for untreated toxin to more than 750 µg per kg body weight for the formalin-treated material. By 48 hours the intravenous ED<sub>50</sub> was increased and at about 9 days, the emetic inducing activity was destroyed almost completely. With smaller concentrations of formalin inactivation took longer. The results of a series of experiments with enterotoxin exposed to 0.3% formalin for different periods of time are summarized in Table 1.

TABLE 1. EFFECT OF 0.3% FORMALIN ON THE EMETIC ACTIVITY FOR MACACA MULATTA MONKEYS OF STAPHYLOCOCCAL ENTEROTOXIN B (INTRAVENOUS INJECTION)

Dose, <sup>a</sup> / µg/kg body wt	Emetic Response, <sup>b</sup> / per cent Hours of Inactivation					
	0	8	24	48	96	144
0.1	-	33	67	100		
0.3 - 0.6	82	33	60	36	50	0
0.9	67			67		
1.8 - 3.0	80	67	100	62		
6.0 - 12	100		50	50	50	0
30	100	100	100	88		
60	100		100	78	100	100

a. The intravenous ED<sub>50</sub> (emetic dose) for enterotoxin B in rhesus monkeys is 0.26 µg per kg body weight.

b. 2 to 14 animals per group.

It is apparent that formaldehyde impairs both the immunochemical and biological activity of enterotoxin B. The rate of change for various properties of the toxin varies, however. Antigenic changes occur quite rapidly and the lethal effect disappears more rapidly than the emetic properties. It seems likely that the difference among these properties may be quantitative rather than qualitative. As the complex reaction between the aldehyde and the susceptible groups on the molecule proceeds and combination of formaldehyde with the  $\text{NH}_2$  groups and the subsequent formation of cross-linkages occurs, more of the antigenic and toxigenic groups become inactive. The importance of the  $\text{NH}_2$  groups on the molecule is indicated by the observation by Dr. Schantz, that treatment of enterotoxin B with  $\text{HNO}_2$  caused complete and immediate inactivation of the toxin. Studies are planned to associate chemical changes with the observations reported here.

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